Chitosan conjugated cyclodextrin nanocomposite loaded with antibiotic-adjuvant combinations remediates multi-drug resistant *Staphylococcus aureus* infection in CD-1 mice model of bovine mastitis

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Supplementary information

Supplementary information 1: Assessment of CF, CPZ, and TA for antibacterial synergism

A three-dimensional checkerboard assay, as previously described by Stein *et al*., was conducted to assess the antibacterial efficiency of the combination (CF, TA, and CPZ) [1]. CF, CPZ, and TA concentrations were selected based on the pre-determined MICs against Sa1158c. Ten-twofold, six-twofold, and seven-twofold serial dilutions of CF, CPZ, and TA, respectively, were performed in a 96-well plate. Ten µL of Sa1158c culture maintained at 0.5 McFarland standard (1.5 × 10^8 cells/ mL) in MHB broth was added further. The plates were incubated at 37 °C for 18 h, and 30 µL of resazurin solution (0.5% of 100X resazurin in PBS buffer (pH-7.4)) was added to each well, and the plates were incubated for 2 h under mild shaking. The fluorescent intensity (530/590 nm) was measured using a plate reader (SpectraMax-i3X, Molecular Devices, USA).

The fractional inhibitory concentration index (FICI) was determined using the following formula:

$$FICI_{CF/TA/CPZ} = (MIC_{CF(combination)}/MIC_{CF(alone)}) + (MIC_{CPZ(combination)}/MIC_{CPZ(alone)}) + (MIC_{TA(combination)}/MIC_{TA(alone)}$$

FICI < 0.8, 0.8 < FICI < 4, and FICI ≥ 4 were considered synergism, additive or indifference, and antagonism effects, respectively.

Supplementary information 2: Physicochemical characterization of the particles

An attenuated total reflectance-Fourier transform infrared (ATR-FT-IR) (ALPHA-P, Bruker, Billerica, MA, USA) was used to assess the surface functional groups of the particles [2]. Five microliters of the particle suspension were dropped on the ATR probe and allowed to dry. A wavelength range of 400–4000 cm^{-1}, with a resolution of 4 cm^{-1}, and 32 scans were used to obtain the FT-IR spectrum. Qualitative analysis was performed using the OMNIC 8.2.0.387 software.

The FTIR spectra of CPZ-CD-TA and CH Np-CF are provided in **Figure 1a**. The absorbance at 1151 cm^{-1} in CH Np-CF is characteristic of the asymmetric vibration of the b-(1-4) glycosidic bond of chitosan. The low-intensity peaks at 2935 and 2887 cm−1 are attributed to stretching vibrations of methylene groups in the polymeric chain. The peaks at 1072 cm^{-1} and 1531 cm^{-1} represent C-O-C- stretching vibrations and $–NH_2$ bending vibration peaks, respectively. The peak at 1030 cm^{-1} shows the characteristic of P=O stretching vibration from phosphate groups, while the peak at 1628 cm^{-1} is attributed to the electrostatic cross-linking between the phosphate group of TPP and the ammonium group of chitosan, suggesting the formation of CH Np [3, 4]. CF in CH Np-CF was evident from the additional signals in 1763, 1635, 1384, and 1287 cm^{-1} corresponding to C=O stretching, $-NH_2$ bending, C-N stretching, and C-O stretching, respectively. CD Np in CPZ-CD-TA was evident from the characteristic bands of saccharides: 2932 cm^{-1} (C-H stretching vibration), 1651 cm^{-1} (O-H bending vibration), and 1154 cm^{-1} (C-O vibration) [5]. The signal at 857 cm^{-1} corresponded to the α-type glycosidic bond, characteristic of CD Np formed by glucopyranose units through the α-1,4-glycosidic bond [5]. The peak at 2973 cm^{-1} corresponds to the anti-symmetric vibration of a methyl group, indicating the existence of the hydroxypropyl group in CD Np [5]. CPZ in CPZ-CD-TA was evident from the signals 1606 and 1651 cm^{-1} (phenyl rings), 1510 cm^{-1} (C-H deformation), and 752 cm^{-1} (aromatic C-H bending) [6]. TA showed an adsorption band at approximately 3000–3500 cm^{-1} belonging to the stretching vibration of O-H groups. The absorption band at 1187 cm^{-1} belongs to the bending vibration of O-H groups. The skeleton vibration band of benzene rings appeared at 1443–1704 cm^{-1} [7].

A Scanning Electron Microscope (SEM) (FEG Quanta 450 Field Emission Scanning Electron Microscope, USA) was used to analyse the surface morphology of the particles [8].

Suspensions of particles (50 µg/mL) were dropped onto aluminum mounts, dried at room temperature, and coated with platinum before acquiring SEM images. ImageJ software was used to measure the size of particles. The hydrodynamic size and surface charge of the particles (50 µg/mL) were measured by Dynamic Light Scattering (DLS) (NanoBrook Omni instrument, Brookhaven, USA) at 25 °C [7]. The particles were loaded into a pre-rinsed folded capillary cell. A 100 V was applied to measure the zeta potential.

CD Np had a typical nano-sized spherical morphology [9], while CPZ-CD-TA showed aggregation of particles forming a chain-like arrangement **(Figures 1b-g)**. The size of CPZ-CD-TA ranged between ~120-260 nm. DLS studies indicated the hydrodynamic size of CD Np and CPZ-CD-TA to be ~135 nm and a zeta potential of ~-11 mV and ~-9.50 mV, respectively **(Table 1)**. The weaker zeta potential might have led to the aggregation of these nanoparticles due to the Van Der Waals attractive forces [7]. CH Np was uniformly distributed and had a typical spherical structure with a size range between ~114-159 nm. Similarly, CH Np-CF showed a spherical morphology and was of variable sizes (~92-229 nm).

Supplementary information 3: *In vitro* antibacterial efficiency of particles

The *in vitro* antibacterial efficiency of the particles was determined using the broth microdilution method [10]. Briefly, the particles (250 µg/mL) were subjected to ten-twofold serial dilution in 100 µL of MHB media in a sterile 96-well plate. Bacterial culture of 10 µL taken from cell suspension with OD adjusted to 0.5 McFarland standard (1.5×10^8 cells/mL) was added to the wells. The plate was incubated at 37 °C for 18 h. The colony-forming units (CFU) were enumerated using a drop plate culturing method, as detailed earlier [10, 11]. For this, 10 µL from the treated wells were subjected to seven-fold serial dilutions in 100 µL of PBS (1X, pH 7.4) in a new 96-well plate.

Subsequently, 10 µL of the cell suspension (from 10^1, 10^3, 10^5, and 10^7 dilutions) was dropped onto one end of the rectangular TSA plates and allowed to flow down vertically. These plates were further incubated at 37 °C for 18 h, and the CFU in respective lanes were counted manually.

Supplementary information 4: Efficiency of NeACT against internalized *S. aureus* in epithelial cells

The cytotoxicity of NeACT was tested in Caco-2 cells [12]. Briefly, the Caco-2 cells were cultured in a 96-well plate (2×10^4 cells/well) until confluent and exposed to increasing concentrations of the particles (3.90-250 µg/mL) suspended in DMEM media. After 24 h of incubation, resazurin of 50 µg/mL (prepared with DMEM) was added and incubated for 4 h. The fluorescence was measured at 530/590 nm using a plate reader. Cells without treatment and cells treated with silver nitrate ($AgNO_3$) were considered as negative and positive controls, respectively.

The efficiency of NeACT to target internalized *S. aureus* in epithelial cells (Caco-2) was determined by a pre-established protocol with modifications [8, 13]. Confluent Caco-2 cells (2×10^4 cells/well) were exposed to Sa1158c culture maintained at 1.5×10^8 cells/mL in a 96-well plate and incubated for an hour. The cells were washed using PBS (4 ˚C) and subjected to gentamicin (10 µg/mL) for 30 min. The extracellular gentamicin was removed by washing, followed by incubation with DMEM for 4 h to establish the intracellular infection model. The particles at incremental concentrations (3.90-15.62 µg/mL) were added, and the plates were incubated for 24 h in a cell culture humidified incubator at 37 °C, with 5% CO_2. Subsequently, the cells were washed using PBS and lysed using 0.5% (v/v) of Triton X-100. CFU of viable intracellular bacteria were enumerated using the drop culture method as detailed earlier in section 5.2.8. Sa25923 was used as a reference strain, and cells infected with bacteria without treatment were considered as controls.

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